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<b>(54) Title:</b> EXTRACTION OF TRIGLYCERIDES FROM MICROORGANISMS			
<b>(57) Abstract</b> Oil is extracted from oil containing microorganisms by disintegrating the microorganisms and contacting them in the presence of a water content of at least 70 % by weight of that originally present in the cellular material with a water immiscible solvent for the oil, separating the solvent from the microorganisms and recovering the oil from the solvent.			

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## EXTRACTION OF TRIGLYCERIDES FROM MICROORGANISMS

This invention relates to an extraction process.

Vegetable oils can be extracted from dried plant seeds using pressure. This in general leaves vegetable oil in the crushed plant tissue and this may be extracted using a water-immiscible organic solvent, for example hexane.

It is possible to extract microbial oils from whole microbes by hexane extraction. The microbial material must be dried usually to a water content of less than 5% (w/w) before the oil can be effectively extracted by hexane. Not only does this involve a considerable consumption of energy because, in general, microbial cells contain at least 70% for example 80 to 95% (w/w) water based on their non-oil content, but we have also found that sensitive oils for example oxygen or heat sensitive oils may undergo chemical reactions in the drying process.

In alternative methods of extracting oils from microorganisms, water-miscible solvents, for example isopropanol, may be used. Such extractions are in general less selective than those with non-polar solvents and a wide range of other cellular components may be extracted in addition to the oils, for example phospholipids and cell wall components. Evaporation of the water-miscible solvent leaves a residue which must therefore be purified further.

By "oils" is meant materials which are liquid at the extraction temperature and which are sparingly soluble in water.

In general oils recovered from microorganisms are

triglycerides.

This invention comprises a process in which an oil is extracted from oil containing microorganisms which comprises disintegrating the microorganisms and

5       contacting them in the presence of a water content of at least 70% by weight of that originally present in the cellular material and preferably in the presence of substantially all of the original water content of the microorganisms with a water immiscible solvent for the

10      oil, separating the solvent from the microorganisms and recovering the oil from the solvent.

The invention also comprises a process for extracting a triglyceride from microbial matter containing it in which the triglyceride is released from

15      the cells by pressure homogenisation and the released triglyceride is then extracted by contacting the microbial matter with a water-immiscible solvent for example an alkane suitably having 4 to 12 and preferably 5 to 8 carbon atoms, for example cyclohexane or

20      preferably hexane. The invention avoids the requirement for dehydrating the microbial matter whilst permitting a good selectivity of extraction into the water-immiscible solvent to be secured.

It is preferred that the microorganism should be

25      disintegrated in the presence of an aqueous culture medium in which it has been cultured, as this avoids the need for separating the microbial matter from the culture medium prior to the process. Typically the organism will constitute 5% to 20% by volume of the culture medium. The

30      same solvent may be re-used two or more times for extracting successive quantities of disintegrated

organism containing triglycerides, thus increasing the triglyceride content of the solvent. This leads to economies in recovering the triglyceride from the solvent by evaporation. Such processes may, if desired, be 5 conducted by countercurrent extraction. Suitably, this may be carried out by using a solvent which is less dense than the phase containing the disintegrated microorganism and feeding it at a lower level than a phase which contains the disintegrated microorganism to a vessel in 10 which extraction occurs. The vessel may be provided with baffles, restricting vertical flow, which define contact zones between the baffles and in which means is provided for stirring material in the contact zones.

If desired, separated solvent containing the 15 triglyceride may be used together with fresh solvent to extract previously unextracted microorganisms. The solvent may be separated by centrifuging. The solvent may suitably be a hydrocarbon, for example an alkane which suitably has 4 to 12 and preferably 6 to 10 carbon 20 atoms and is suitably hexane.

The organisms may be disintegrated by enzyme cell disruption using cell wall lytic enzymes, mechanical methods such as bead milling, colloid milling, disruption by pressure release, impinging jets, ultrasonication and 25 preferably using high shear mixing and/or high pressure homogenisation. The disruption should be sufficient to disrupt the cell wall, thus enhancing the access of the solvent to the triglycerides contained within the cell. Any form of pressure homogeniser may be employed for this 30 purpose.

In general in solvent extraction processes, small

quantities of solvent are used compared with the material which is to be extracted. This has the advantage of achieving a high concentration of the desired solute in the solvent phase but when microbial materials are extracted in this way there is a tendency for organic matter present to act as an emulsifying agent, leading to the formation of a solvent-in-water emulsion of high stability. In order to reduce the stability of such emulsions, it may be necessary to add demulsifying agents thus enabling the emulsion to be separated suitably using high gravity conditions. However, the demulsifying agent may contaminate the product which it is desired to separate and involves additional cost. Contamination may be particularly undesirable if the demulsifier is toxic.

We have found that by operating in the presence of high concentrations of solvent sufficient to form a water-in-solvent dispersion, the dispersion is less stable and may be more readily separated in the absence of a demulsifying agent, for example by gravity settling or use of a centrifuge. It is desirable in this case to increase the triglyceride content of the solvent by extracting successive quantities of microbial material with it.

This invention therefore comprises a process as aforesaid in which the disintegrated microorganisms are contacted with a continuous phase of the solvent. It is desirable that the ratio of solvent phase to other matter present during the extraction step should be at least 1 to 1, preferably at least 1.5 to 1 and more preferably at least 2 to 1 and suitably at most 10 to 1 and preferably at most 1 to 1.

EXAMPLE 1

A strain of *Mortierella alpina* was grown in batch culture in an aqueous medium on a mixture of glucose and yeast autolysate to give a culture containing 54.8 g/l of 5 cells containing 24% w/w of a triglyceride oil. The fatty acid composition of this oil was determined to be 18.6% w/w 5,8,11,14-eicosatetraenoic acid (arachidonic acid).

The cell suspension was dispersed using a high shear 10 mixer (Ultra Turrax, IKA) then fed to a high pressure homogeniser (Rannie Lab homogeniser, APV) operating at a pressure of 400 bar. The resulting cell homogenate was then first mixed with a demulsifying agent (Armogard D5390, Akzo-Nobel, Littleborough) to give a concentration 15 of the demulsifier of 2000 ppm. This aqueous phase was then contacted (at ambient temperature and neutral pH) with hexane by high shear mixing (Ultra Turrax, IKA) to ensure good phase contact. A phase ratio of 3:1 aqueous to solvent was used. The resulting emulsion was 20 separated by centrifugation at 3000 g for 3 minutes. The separated hexane phase was purified from residual fermentation antifoam by passing through a silica gel column (Waters Sep-pak) and evaporated to give the purified triglyceride oil. This oil was analysed for 25 5,8,11,14-eicosatetraenoic acid content by Gas Chromatography. The overall triglyceride yield from hexane contact of the aqueous phase was 65.7% based on the initial oil content of the microbial cells. The 5,8,11,14-eicosatetraenoic acid content of the oil was 30 28.1% w/w.

In comparison a sample of fermenter culture was

taken and the cells recovered by filtration. The collected cells were dried by freeze drying then mixed with hexane. 5 g of dried cells were contacted with 25 ml of hexane. The solvent was contacted with the 5 cells for 60 minutes at ambient temperature before the solvent extract was recovered by filtration of the suspension. The solvent was purified of residual fermentation antifoam using a silica gel column (Waters Sep-pak) and evaporated to give a purified triglyceride oil. The overall triglyceride yield was 59% based on the 10 initial oil content of the microbial cells. This was analysed for 5,8,11,14-eicosatetraenoic acid by Gas Chromatography giving a concentration of 7.1% w/w.

This shows that considerable breakdown of the 15 5,8,11,14-eicosatetraenoic acid had occurred during the extraction of dried cells compared to the extraction from homogenised whole cell culture.

Oil yield was higher from extraction from high pressure homogenised whole cells compared to freeze dried 20 cells and the oil quality was higher in terms of the recovered 5,8,11,14-eicosatetraenoic acid.

#### **EXAMPLE 2**

A strain of *Mortierella alpina* was grown in batch culture in an aqueous medium on a mixture of glucose and 25 yeast autolysate to give a culture containing 46.0 g/l of cells containing 51.0% w/w of a triglyceride oil. The fatty acid composition of this oil was determined to be 30 25.1% w/w 5,8,11,14-eicosatetraenoic acid (arachidonic acid).

The cell suspension was dispersed using a high shear mixer (Ultra Turrax) then fed to a high pressure

homogeniser (Rannie) operating at a pressure of 400 bar. This aqueous phase was then contacted (at ambient temperature and neutral pH) with different quantities of hexane by high shear mixing (Ultra Turrax) to ensure good phase contact. Phase ratios of 1:1, 1:1.5, 1:2, 1:2.5, and 1:3 aqueous to solvent were used. The resulting mixed phases were separated by centrifugation in graduated test tubes at 3000 g for 3 minutes and the recovery of the initial solvent phase measured.

10 TABLE 1

	Phase ratio (aqueous:solvent)	Solvent recovery (%)
	3:1	4
	2:1	15
	1:1	88
15	1:1.5	95
	1:2	97
	1:2.5	101
	1:3	100

20 In the case of the sample prepared using a phase ratio of 1:2 aqueous:solvent the solvent phase was recovered and the aqueous phase recontracted with sufficient hexane solvent to give a phase ratio of 1:2 aqueous:solvent. As for the original extraction a high shear mixer was used to ensure good contact of the phases. The pooled hexane phases were evaporated and analysed for total triglyceride content and of 5,8,11,14-eicosatetraenoic acid. The total triglyceride yield was

76% and the 5,8,11,14-eicosatetraenoic acid content of the oil was 29.2% w/w.

**EXAMPLE 3**

5       A strain of *Thraustochytrium* was grown in batch culture in an aqueous medium on a mixture of glucose, yeast extract, peptone and 20 g/l Na<sub>2</sub>SO<sub>4</sub> to give a culture containing 15 g/l of cells containing 30% w/w of a triglyceride oil. The composition of the fatty acids of this oil was determined to be 10% w/w 7, 10, 13, 16, 19-  
10       docosahexaenoic acid (DHA).

The cell suspension was passed twice through a high pressure homogenised (Niro-Soavi Panda Laboratory homogeniser) operating at a pressure of 500 bar.

15       This aqueous phase was then contacted (at ambient temperature and neutral pH) with three times the aqueous phase volume of hexane by high shear mixing (Ultra Turrax, IKA) to ensure good phase contact.

The resulting mixed phases separated under gravity and the solvent phase recovered and retained.

20       The aqueous phase was contacted again with hexane (volume equal to three times the aqueous phase volume) and the hexane extract separated by gravity settling.

The pooled hexane phases were evaporated and analysed for total triglyceride content and of 4, 7, 10, 25       13, 16, 19- docosahexaenoic acid (DHA).

The total triglyceride yield was 91% and the 4, 7, 10, 13, 16, 19- docosahexaenoic acid (DHA) content of the oil was 8.7% w/w. The peroxide value (PV) of the oil was measured to be less than 5 milliequivalents of active  
30       oxygen per kg.

95TJL05S - MS - 2 July 1996

## CLAIMS

- 1 A process in which an oil is extracted from oil containing microorganisms which comprises disintegrating the microorganisms and contacting them in the presence of a water content of at least 70% by weight of that originally present in the cellular material with a water immiscible solvent for the oil, separating the solvent from the microorganisms and recovering the oil from the solvent.
- 2 A process in which an oil is extracted from oil containing microorganisms which comprises disintegrating the microorganisms and contacting them in the presence of substantially all of their water content with a water immiscible solvent for the oil, separating the solvent from the microorganisms and recovering the oil from the solvent.
- 3 A process as claimed in Claim 1 or 2 in which the oil is a triglyceride.
- 4 A process as claimed in any preceding claim in which the oil is oxygen- or heat- sensitive.
- 5 A process as claimed in any preceding claim in which the microorganism is disintegrated in the presence of an aqueous culture medium thereby forming an oil containing medium and contacting the said medium with the solvent.
- 6 A process as claimed in any preceding claim in which the disintegrated organisms are repeatedly contacted with solvent thereby extracting successive quantities of oil therefrom.

- 7 A process as claimed in any preceding claim in which previously unextracted disintegrated microorganisms are extracted with solvent which contains triglyceride extracted from disintegrated microorganisms.
- 8 A process as claimed in Claim 7 which is carried out by counter-current extraction.
- 9 A process as claimed in Claim 8 in which the solvent is less dense than the phase containing the disintegrated microorganism and is fed to a vessel in which extraction occurs at a lower level than the phase containing the disintegrated microorganism.
- 10 A process as claimed in Claim 8 in which the solvent is more dense than the phase containing the disintegrated microorganism and is fed to a vessel in which extraction occurs at a higher level than the phase containing the disintegrated microorganism.
- 11 A process as claimed in Claims 9 or 10 in which the vessel is provided with baffles restricting vertical flow which define contact zones between the baffles and means for stirring material in the contact zones.
- 12 A process as claimed in any preceding claim which comprises separating the solvent by centrifuging.
- 13 A process as claimed in any preceding claim in which the disintegrated microorganisms are contacted with a continuous phase of the solvent.
- 14 A process as claimed in Claim 12 in which during extraction the ratio of solvent phase to other matter present is at least 1 to 1 preferably 1.5 to

1, and more preferably at least 2 to 1 and is suitably at most 10:1 and preferably 5:1.

15 A process as claimed in any preceding claim in which the cells are disintegrated by pressure homogenisation.

16 A process as claimed in any preceding claim in which the solvent is a hydrocarbon.

17 A process as claimed in Claim 15 in which the hydrocarbon is hexane.

## INTERNATIONAL SEARCH REPORT

Intern'l Application No  
PCT/GB 96/01703A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12P7/64 C11B1/10 C12N1/06 C12N1/00

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## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12P C11B C12N

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 8413 Derwent Publications Ltd., London, GB; Class D16, AN 84-077725 XP002018398 & JP,A,59 028 480 (NISHIYAMA GUM KK) , 15 February 1984 see abstract --- A WO,A,92 13086 (MARTEK CORP) 6 August 1992 see page 11; claims --- A PATENT ABSTRACTS OF JAPAN vol. 008, no. 114 (C-225), 26 May 1984 & JP,A,59 028480 (KOUGIYOU KAIHATSU KENKYUSHO:KK;OTHERS: 01), 15 February 1984, see abstract ---	1-3,5,7, 16 1 1
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## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB,A,1 089 093 (THE BRITISH PETROLEUM COMPANY) 1 November 1967 see page 5, line 9 - page 6, line 95 ---	1
A	EP,A,0 522 470 (IDEMITSU PETROCHEMICAL CO) 13 January 1993 see claims -----	1

1

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat'l Application No  
PCT/GB 96/01703

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9213086	06-08-92	AU-B-	661674	03-08-95
		AU-A-	1235592	27-08-92
		BR-A-	9205519	01-03-94
		CA-A-	2101273	25-07-92
		EP-A-	0568608	10-11-93
		IL-A-	100732	29-06-95
		JP-T-	6505384	23-06-94
		NZ-A-	241358	27-09-94
		OA-A-	9909	15-09-94
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GB-A-1089093		BE-A-	653910	02-04-65
		DE-A-	1442058	06-02-69
		NL-A,B	6411478	05-04-65
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EP-A-0522470	13-01-93	JP-A-	5308979	22-11-93
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		AU-A-	1946792	21-01-93
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